

## 9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

### 9.1 Availability of Other *In Vitro* ER TA Data

Some of the peer-reviewed publications identified during the initial literature search for *in vitro* ER TA studies were not abstracted for inclusion in this BRD. The reasons for not abstracting these publications include:

- The studies lacked appropriate qualitative and/or quantitative test data;
- The test substances were not adequately identified, or were undefined mixtures; or,
- The publications contained insufficient information about the test method used.

NICEATM published a formal request in the *Federal Register* (Vol. 66, No. 57, pp.16278 – 16279) for unpublished *in vitro* ER TA data and/or information from completed studies using or evaluating *in vitro* ER TA assays. Two submissions were received in response to this request, one from Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan, and the other from Xenobiotic Detection Systems, Inc., Durham, North Carolina. The data from these unpublished submissions are included in **Appendix D**, which also contains the *in vitro* ER TA data from the published articles considered in this BRD.

A number of companies involved in pharmaceutical discovery and development routinely use *in vitro* ER TA assays to screen substances for their potential estrogenic activity. However, these data are not in the public domain and were not provided to NICEATM.

While every effort was made to include all available, pertinent *in vitro* ER TA data in this BRD, NICEATM recognizes that some data may have been inadvertently excluded.

### 9.2 Conclusions from Other Scientific Reviews of *In Vitro* ER TA Methods

To date, no independent peer reviews of *in vitro* ER TA assays have been conducted. However, two recent workshops addressed the use of these assays as potential endocrine disruptor screening methods. The strengths and limitations of *in vitro* ER TA assays were discussed at both workshops, but no efforts were made to evaluate the reliability and performance of the assays. The conclusions from these workshops are summarized below.

### 9.2.1 1996 Endocrine Disruptor Screening Methods Workshop

*In vitro* ER TA and cell proliferation assays were discussed at an Endocrine Disruptor Screening Methods Workshop held in July 1996 at Duke University in Durham, North Carolina. Gray et al. (1997) edited the proceedings of this workshop, which was cosponsored by the U.S. EPA, the Chemical Manufacturers Association (CMA), and the World Wildlife Fund (WWF).

The proceedings briefly discussed *in vitro* ER TA assays that use MCF-7 cells, a cell line derived from human mammary carcinoma cells. In these assays, ER is expressed endogenously, while a reporter gene that is linked to an ER-inducible response element is transiently transfected into the MCF-7 cells. This type of *in vitro* ER TA assay is routinely used to measure ER-induced TA. In the proceedings, it was noted that luciferase activity appears to be a more sensitive indicator of TA than CAT expression.

A variation of the MCF-7 assay described in the proceedings uses a chimeric receptor in which the hER ligand binding domain is fused to Gal4. MCF-7 cells are transiently co-transfected with a vector containing the cDNA for the chimeric receptor and a vector containing a Gal4-controlled luciferase construct. This assay was originally developed to differentiate substances that elicit estrogen activity through phosphorylation of the ER from those that activate the ER through binding. While this assay is not widely used, a variety of chemical classes, including organochlorines, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, phthalates, and alkylphenols, have been tested by the few laboratories that employ it. Gray et al. (1997) indicated that “these assays ... have relatively high sensitivity.”

An *in vitro* ER TA assay using stably transfected MCF-7 cells was also discussed at the workshop. The discussion focused on one stably transfected MCF-7 cell line, MVLN, which endogenously expresses hER and contains transfected genes that encode luciferase, linked to a vitellogenin promoter. As described in the proceedings, the major advantages of this assay over others that measure ER-induced TA include:

- The assay is easier to conduct than ones using transiently transfected cell lines;
- This assay has been standardized in several laboratories; and
- The assay can be used for high-throughput screening.

In addition, Gray et al. (1997) discussed the major advantages and disadvantages of yeast-based *in vitro* ER TA assays. These assays use recombinant, stably transformed yeast (*S. cerevisiae*) cells that contain the complete ER gene, or specific portions of ER from humans or other species of interest, and a reporter gene, typically *lacZ* (which encodes  $\beta$ -galactosidase), linked to an ER-inducible response element.

The major advantages of yeast assays, as described in the proceedings, include:

- The assay is relatively easy to perform because it uses stably transfected cells;
- The incubation time of the test substance is short, ranging from 4 hours to overnight;
- The assay has been automated for high-throughput screening; and
- The assay has been adapted to test chemical mixtures.

In the proceedings, it was noted that the workshop participants did not reach a consensus with respect to the applicability of yeast-based *in vitro* ER TA assays as an endocrine disruptor screening method. The major disadvantages of yeast-based *in vitro* ER TA assays identified by the authors include:

- They do not appear to distinguish between agonists and antagonists (e.g., the known ER antagonist, ICI 164,384, is reported to induce TA);
- There may be significant metabolic differences between yeast and mammalian cells that could make it difficult to extrapolate data from these assays to humans;
- The cell wall and chemical transport systems of yeast cells are reported to selectively maintain low intracellular concentrations of some steroid hormones, a phenomenon that may also apply to other types of substances;
- The permeability of the yeast cell wall may differ significantly from that of mammalian cell membranes; and
- Different yeast strains appear to differ in their response to estrogenic substances, the amount of ER expressed, and the uptake of test substances into the cell.

The usefulness of MCF-7 cell proliferation assays, which use cell growth to identify ER agonists and antagonists, was discussed also at the workshop. Although the assay appeared to be very

sensitive, a number of factors could affect the utility of the assay for the large-scale screening of substances. One major concern noted in the proceedings is the potential difficulty of standardizing MCF-7 cell culture procedures, which must change over time to accommodate changes in MCF-7 cells over time. Another concern is that frozen MCF-cells generally require an adaptation period (sometimes up to three months) after thawing before exhibiting their full response to estrogens. Also, the duration of the assay, about six days, is considered somewhat long for an *in vitro* assay. One other concern is the potential for false positive and false negative results from substances that promote cell growth through other mechanisms, or that are cytotoxic.

### **9.2.2 1997 Workshop on Screening Methods for Detecting Potential (Anti-) Estrogenic/Androgenic Chemicals in Wildlife**

In March 1997, the U.S. EPA, the CMA, and the WWF cosponsored a workshop in Kansas City, Missouri, that addressed the use of “gene expression” assays as a type of *in vitro* screening method for detecting potential (anti-)estrogenic substances in wildlife. Ankley et al. (1998) edited the proceedings of this workshop.

The major advantages described by the authors for using gene expression assays as endocrine disruptor screens for wildlife include:

- Assays that use eukaryotic cell lines can distinguish between agonists and antagonists;
- The assays are amenable to automation using microtiter plates, which would allow for the rapid processing of large numbers of samples; and
- The methods are amenable to standardization.

The major disadvantages include:

- The assays require specialized equipment and training;
- Transient transfection of plasmids can be labor-intensive and may contribute to decreased reliability;
- The poor correlation for some substances between yeast-based and mammalian cell-based assays; and

- These assays currently have limited applicability to nonmammalian species, which have been poorly studied with regard to development of suitable reporter gene assays for detection of (anti-)estrogenic substances.

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